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(54) Protein antigen from Mycobacterium tuberculosis

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(57) A 17 KDa protein has been isolated from *M. tuberculosis* (South Indian Isolate -1) and sequenced. This 131 amino acid protein and fragments thereof including amino acids 68-77, 91-101 and/or 107-122, antibodies to the protein and the fragments and DNA encoding the protein or fragments, or DNA hybridisable to such DNA are of interest in the immuno diagnosis, therapy and vaccination in relation to human tuberculosis.

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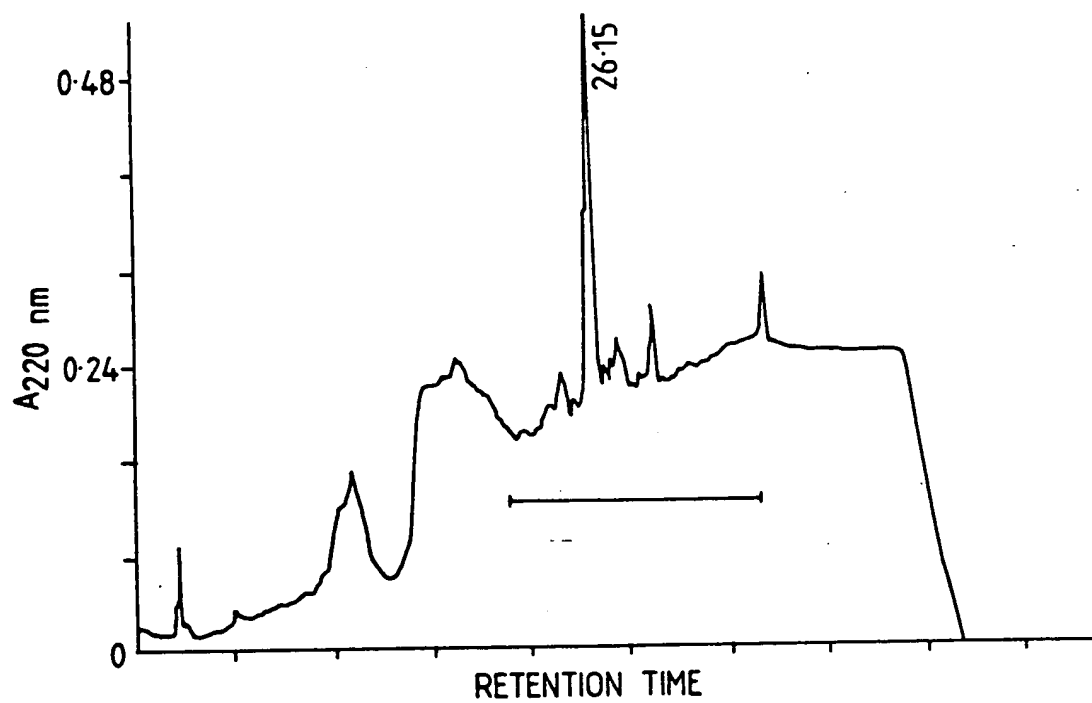
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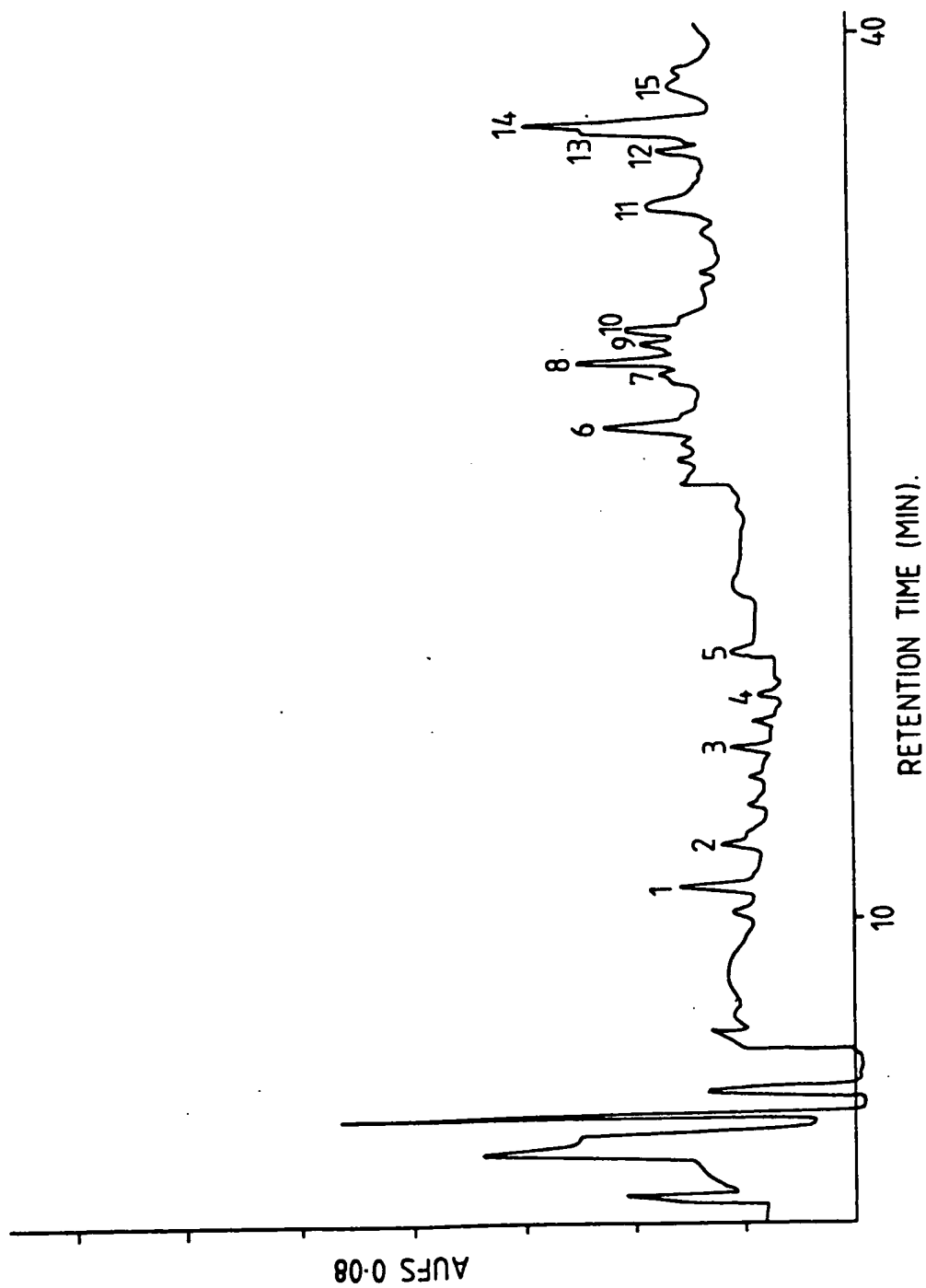
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Fig.1.

Fig.3.

AUFS 0·08

RETENTION TIME (MIN).

Fig.5.


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        5         10        15        20      25
A T T L P V Q R H P R S L F P E F S E L F A A F P S
   30          35        40        45        50
F A G L R P T F D T R E L M R S I Q I T I K L E D E M
 55      60        65        70          75
K G I Y L P V A K H G E L R│S E F A Y G S F V R│T V
   80        85        90        95        100     105
S L P V G A D E D D I│R A T Y D K R Y E V R│D F D G R
 110       115       120       125       130
│A E L P G V D P D C D V C I T R│G I L T V S V C V
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"NEW METHODS FOR DIAGNOSIS OF TUBERCULOSIS"

2. Field of invention

The present invention relates to a novel 17 kDa protein antigen of Mycobacterium tuberculosis (South Indian Isolate SII 1) and certain peptide fragments derived therefrom, and to the use of the said antigen, and of the peptide fragments derived therefrom, in immuno diagnosis, immunotherapy and immuno prophylaxis of human and experimental tuberculosis. The invention also relates to the DNA sequence coding for the said 17 kDa antigen, to DNA sequences coding for the said peptide fragments derived from the 17 kDa antigen and to the DNA and RNA probes constructed on the basis of the protein sequence of the 17 kDa antigen including the sequences of the peptide fragments of the 17 kDa antigen. A major field of use is the use of 17 kDa antigen and peptide fragments thereof in immuno diagnosis of tuberculosis. A further field of use is the use of the 17 kDa antigen or of the said peptide sub structures thereof for the preparation of a vaccine against tuberculosis. A further field of use is the use of the 17 kDa antigen or its sub structure peptides for the detection of T cell proliferation by skin tests or invitro tests in man. This last mentioned field of use is of importance in the possible treatment of human cancer by the boosting of cellular immunity. A further field of use is the use of the 17 kDa antigen or its sub structure peptides for the laboratory production of cellular growth factors and enzymes.

Thus many clinical features of TB are not specific to TB alone and a study in India (source: National Tuberculosis Insitutute, Bangalore, India) revealed that only 30% of the X-ray suspects eventually developed TB although, in many countries including India, all X-ray suspects are put on anti tuberculous chemotherapy.

The microscopy of tuberculous specimen is not easy under field conditions and atleast 10^4 bacilli/ml are required for effective screening. Many tuberculous specimens like cerebro spinal fluids from tuberculous meningitis infrequently contain bacilli. Further, bacteriological culture generally takes 6 to 8 weeks and is expensive as a routine diagnostic measure.

The widely used tuberculin skin test lacks sensitivity and specificity and takes about 3 days for completion. Since chemotherapy of TB requires compliance for atleast 6 months, many patients who are irregular in treatment develop drug resistance and transmit live bacilli. Finally, the traditional BCG vaccination has now been found to give varying levels of protection depending upon geographic regions.

Consequent to these factors, many investigators including the WHO recommend that early diagnosis of TB should be considered as a priority area of research and development.

Table I: M.tuberculosis protein antigens identified
           by N-terminal amino acid sequences.

Investigators	Antigens and N-terminus

Shinnick et al, 1987	65 kDa: R G C R H P V
Yamaguchi et al, 1987	MPB 57: M A K F N I K P L
Pattorroyo et al, 1987	13 kDa: A K V N I
	18 kDa: G D L V G P G A E
	23 kDa: A P K T Y
	30 kDa: F S X P G L
	68 kDa: W M T M T
	77 kDa: G K X I A Y D G A A
Matsuo et al, 1988	30 kDa: F S R P G L P
Ashbridge et al, 1989	19 kDa: E H R V K R G L T V
Baird et al, 1989	10 kDa: A K V N I P K P
Garcia et al, 1989	70 kDa: F Q R I T R Q D L L
Borremans et al, 1989	32 kDa: F S R P G L P

To our knowledge, none of these antigens have been introduced
as immuno diagnostic test products. Two of these antigens
(10 kDa of Baird et al, 1989 and a homologue of 65 kDa
antigen of Shinnick et al, 1987) have been tested for vaccine
potency among experimental animal models where they showed
poor   protection   against   M.tuberculosis   (D.W.   Smith,
University of Wisconsin, USA, personal communication).

protein antigen was present in phage type II virulent
M.tuberculosis and absent in phage type I South Indian low
virulent M.tuberculosis. However, the relationship between
this antigen and virulence is yet to be investigated in
detail.

5. The Invention :

A 17 kDa protein antigen was found to be present among the
isolates of M.tuberculosis. The immuno chemical features of
this antigen are disclosed under this invention.

Thus the present invention relates to:

1. The 17 kDa protein antigen of M.tuberculosis (SII 1) as
defined below and certain sub structures (peptides) of 17 kDa
protein antigen as defined below.

2. A DNA sequence coding for the 17 kDa antigen from
M.tuberculosis (SII 1).

3. A DNA sequence coding for the sub structures (peptides) of
the 17 kDa antigen from M.tuberculosis (SII 1).

9. DNA or RNA probes constructed on the basis of the protein sequence of the 17 kDa antigen or sub structures (peptides) thereof for diagnosis of tuberculosis. Such probes can be constructed by methods known in the art. Labelling of such probes can be done by known methods such as radioisotope incorporation or by non radio-active labelling use for example, biotin.

10. A method of diagnosis of human tuberculosis by interacting body fluids such as serum, CSF, pleural fluids from a patient to be diagnosed with a monoclonal antibody to the 17 kDa antigen or its sub structures (peptides) as defined in paragraph 3 above.

11. A method for diagnosis of human tuberculosis by interacting a body fluid such as serum from a patient to be diagnosed with a 17 kDa protein as defined in paragraph 5 above.

12. A method of diagnosing human tuberculosis by interacting body fluids such as sputum, serum, CSF and pleural fluids from a patient to be diagnosed with a DNA or RNA probe as defined in paragraph 9 above.

13. A method for in vitro detection of human tuberculosis which comprises contacting a sample of a body fluid such as

20. A vaccine against tuberculosis developed on the basis of the 17 kDa antigen or sub structure peptides thereof as disclosed in paragraph 1 above. Such a vaccine can be a product of genetically engineered organisms such as Salmonella, Vaccinia virus etc.

The present invention is exemplified by but not limited to the diagnosis, therapy or prophylaxis of diseases, especially diagnosis of M.tuberculosis infection. Epidemiological screening, forensic investigations, determination of food contaminations, public health surveys, preventive medicine, veterinary and agricultural applications with regard to the diagnosis of infectious agents may be covered by this disclosure.

5.1   Fractionation and purification of 17 kDa antigen.

5.1.1 Crude sonicate antigen.

M.tuberculosis (SII 1) was cultured at 37° C for 2 weeks in

Kirchner's medium and harvested bacilli were killed in cold acetone for 18 h at 4° C. The bacilli were washed thrice with saline and a suspension of 10 mg bacilli in 5 ml saline was sonicated at 40 watts output using a miniprobe of a Branson sonifier. The sonicate was centrifuged at 20,000 x g for 30 min and the supernatant was estimated for protein content (Lowry's method) prior to freeze storage at -70° C.

65% in 60 min. The tryptic map is shown in Fig. 2.

V8 protease map: 30 ug of 17 kDa antigen was treated with staphylococcal V8 protease for 48 h in 0.07% ammonia at 37° C. The molar ratio of the enzyme to substrate was 1:25. The various peptides in the enzyme digest were purified on an HPLC column uder the conditions used for tryptic mapping. The peptide profile is shown in Fig. 3.

5.2.2 Sequence analysis of 17 kDa antigen

The amino acid sequence analysis of the protein and the peptide was done using protein sequencer model 477A (Applied Biosystems Inc., USA) with an on line PTH amino acid analyser. The sample was solubilized in 10% formic acid and fixed onto a polybrene coated (1 mg) TFA treated glass fibre disc and used for sequencing.

The first 18 amino acids from the N-terminal was determined using the whole protein. Based on the amino acid sequences of the tryptic peptides, V8 protease was selected to generate the peptides that could give the overlaps for the tryptic peptides. The alignment of both tryptic and V8 protease peptides gave the complete sequence for the 17 kDa antigen. The details of the overlaps are given in Fig. 4.

5.2.3 Amino acid composition of 17 kDa antigen

The protein has A9, C3, D11, E10, F9, G8, H2, 17, K4, L11,

Electro eluted 17 kDa antigen (10 ug in 100 ul saline) was emulsified with an equal volume of Freund's incomplete adjuvant (FICA) and used for intra peritoneal immunization of 10 BALB/c mice. Serum collected from these mice 30 days after immunization recognized a 17 kDa band in the sonicate antigen of M.tuberculosis SII 1.

Thus this experiment confirms that a polyclonal or monoclonal antibody can be produced in the mouse which recognize the protein structure of 17 kDa antigen or sub structures (peptides) thereof. Such antibodies, in particular the monoclonal antibodies can be used in an antigen detection method like the sandwich ELISA for the detection of the 17 kDa antigen or sub structures thereof among human tuberculosis specimen leading to immuno diagnosis of tuberculosis.

5.3.3 Demonstration that 17 kDa antigen reacts with human TB patient sera.

Sera derived from 24 healthy persons and 20 culture proven TB patients were titrated against the 17 kDa antigen as follows. PVC Dynatech plates were coated with 1 ug/ml PBS of electro eluted 17 kDa antigen for 24 h at 22°C. PBS-BSA blocked plates were then titrated against duplicate (1/200) dilutions of sera which were incubated at 22°C for 2.5 h. Washed plates received anti human IgG HRP conjugate for 1.5 h. Washed plates were then assayed with O-phenylene diamine

formed linear or conformational antibody epitopes, an inhibition of ELISA was carried out in which each of the peptide was assayed against the other five using the mouse antiserum to 17 kDa antibody. The peptides YEVR and ATYDK were mutually inhibitive thus indicating that they were a part of a complete antibody epitope, which was confirmed also by the determination of the complete structure of the 17 kDa antigen as in section 5.2.2, Fig 4. The other four peptides were linear and probably conformational in the presentation of the antibody epitope. Of the said six antibody epitope bearing peptides, the peptides of the following amino acid sequences were synthesized by solid phase method of Merrifield and were found to contain specific and sensitive antibody binding activities :

RATYDKRYEVR : Sensitivity 65%; Specificity 95%

SEFAYGSFVR : Sensitivity 66%; Specificity 95%

The antibody epitope mapping as described has thus indicated that defined sub structures or peptides of 17 kDa antigen can be synthesized and used for the immunodiagnosis of human tuberculosis in micro ELISA.

5.3.5 Demonstration that 17 kDa antigen is lympho proliferative

Peripheral blood lymphocytes (PBL) from healty donors and TB patients were fractionated and 2×10^6 cells were cultured in

elaborate several cellular growth and differentiation factors which contribute to the vaccine effect of 17 kDa antigen or its sub structures, the 17 kDa antigen can be used at the first instance as a vaccine against TB and also for non-specifically boosting cellular immunity.

Figure 6 shows the primary structure of the 17 kDa antigen from M.tuberculosis containing the biologically active regions, although similar activity need not be ruled out in the unmarked regions.

6. Discussion and summary of test results

The present invention describes the immunochemical properties of a novel 17 kDa protein antigen from M.tuberculosis (SII 1 strain). M.tuberculosis causes tuberculosis worldwide among 16 million people. Because of inadequacy of the diagnostic procedures available now the disease has not yet been eradicated. The focus of research in recent years has been the development of immuno diagnostic methods for detecting TB at an early stage as well identification of suitable candidates for vaccination since the traditional BCG vaccine has given only a partial protection against TB.

The studies described in this invention show that a novel 17 kDa antigen derived from M.tuberculosis has antigenic activity. Firstly, it was found to be unique for the South Indian strains of M.tuberculosis. Secondly, its antigenic

7. Figure legends.

Fig. 1. HPLC analysis of the electro eluted 17 kDa protein antigen from M. tuberculosis (SII 1).
HPLC conditions: RP 18 column (LKB, 10 um pore size), A: 0.1% TFA in water, B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% B in 40 min, Sensitivity: 0.08,220 nm.

Fig. 2. Tryptic peptides of 17 kDa protein antigen from M.tuberculosis (SII 1) fractionated by HPLC on RP 18 (LKB,10 um pore size).
Fractionation: A: 0.1% TFA in water, B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% B in 60 min, Sensitivity: 0.08, 220 nm.
Amino acid sequence: Sequence determined are drawn against each peptide.

Fig. 3. V8 protease peptides of 17 kDa protein antigen from M.tuberculosis (SII 1) fractionated by HPLC on RP 18 (LKB, pore size 10 um).
Fractionation: A: 0.1% TFA in water; B: 0.085% TFA in 70% aceto nitrile, Gradient: 0 to 65% in 60 min, Sensitivity: 0.082,220 nm.

Fig. 4. The primary structure of the 17 kDa antigen of M.tuberculosis (SII 1) showing the alignment of peptides. Trp: Trypsin, V8: Staphylococcus aureus V8 protease. Superscript arrows denote the amino acid sequence obtained with the whole protein.

(Single letter code used for amino acids).

Fig. 5. The primary structure of the 17kDa antigen from M.tuberculosis (SII 1) showing the biologically active regions.
AA 68 to 77: Antibody and T cell epitopes present.
AA 91 to 101: Antibody epitope present.
AA 107 to 122: Two T cell epitopes present.

CLAIMS

1. The protein of the structure:

1   40
ATTLPVQRHPRSLFPEFSELFAAFPSFAGLRPTFDTRELM

                                          80
5 RSIQITIKLEDEMKGIYLPVAKHGELRSEFAYGSFVRTVS

                                                     131
LPVGADEDDIRATYDKRYEVRDFDGRAELPGVDPDCDVCITRGILTVSVCV

2. Peptides of the structure:

SEFAYGSFVR

10 RATYDKRYEVR

AELPGVDPDCDVCITR

and fragments of the protein according to claim 1 which

include one or more of these peptide sequences.

3. A DNA sequence coding for the protein defined

15 in claim 1.

4. A DNA sequence coding for peptides defined in

claim 2.

5. A DNA or RNA hybridization probe, constructed

on the basis of the proteins or peptides defined in claim 1

20 or 2 or on the basis of DNA sequences according to claim 3

or 4, said probe being optionally labelled.

6. An antibody to a protein or peptide as defined

in claim 1 or 2.

7. An antibody according to claim 6 which is a

25 polyclonal or a monoclonal antibody, said antibody being

optionally labelled.

16. An _in vitro_ method for detection of human
tuberculosis, which comprises contacting a sample of a body
fluid such as sputum, CSF, pleural fluid or serum from a
patient with a polyclonal antibody according to claim 7 in
5 labelled form.

17. A protein or peptide according to claim 1 or 2
for use in a method of treatment of the human or animal
body in immuno-diagnosis, therapy or vaccination in
relation to human tuberculosis.

10   18. A protein or peptide according to claim 1 or 2
for use in a method of treatment of the human or animal
body for producing mammalian polyclonal antibodies or
monoclonal antibodies in mice for use in an
immunodiagnostic method like sandwich ELISA which detects
15 the protein according to claim 1 among human tuberculous
specimens.

19. A protein or peptide according to claim 1 or 2
for use in a method of treatment of the human or animal
body for the detection of antibodies among tuberculous
20 specimens by serological methods for early detection of
tuberculosis.

20. A protein or peptide according to claim 1 or 2
for use in a method of treatment of the human or animal
body for the detection of T cell proliferation in a
25 tuberculous specimen for obtaining immuno diagnosis through
skin tests or obtaining a candidate for vaccine against

as hereinbefore described.

31.   A method according to any one of claims 13 to 16 substantially as hereinbefore described.

32.   A protein or peptide according to any one of claims 17 to 20 substantially as hereinbefore described.

33.   A use according to any one of claims 21 to 24 substantially as hereinbefore described.